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<p>(21) International Application Number: PCT/US00/07336</p> <p>(22) International Filing Date: 20 March 2000 (20.03.00)</p> <p>(30) Priority Data: 60/126,539 26 March 1999 (26.03.99) US 09/286,033 5 April 1999 (05.04.99) US</p> <p>(71) Applicant (<i>for all designated States except US</i>): REGENERON PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (<i>for US only</i>): SURI, Chitra [IN/US]; 21 Tappan Landing Road, Tarrytown, NY 10591 (US). YAN-COPOULOS, George, D. [US/US]; 1519 Baptist Church Road, Yorktown Heights, NY 10598 (US). THURSTON, Gavin [US/US]; 1839 Tenth Avenue, San Francisco, CA 94122 (US). MCDONALD, Donald [US/US]; 67 Parker Avenue, San Francisco, CA 94118 (US).</p> <p>(74) Agents: PALLADINO, Linda, O.; Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US) et al.</p>		<p>(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: MODULATION OF VASCULAR PERMEABILITY BY MEANS OF TIE2 RECEPTOR ACTIVATORS</p> <p>(57) Abstract</p> <p>The present invention provides for a method of decreasing or inhibiting vascular permeability and/or plasma leakage in a mammal comprising administering to the mammal a TIE2 receptor activator. The invention also provides for a method of decreasing or inhibiting vascular permeability and/or plasma leakage in a mammal comprising administering to the mammal an Ang-2 inactivator such as an anti-Ang-2 neutralizing antibody.</p>		

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MODULATION OF VASCULAR PERMEABILITY BY MEANS OF TIE2 RECEPTOR ACTIVATORS

This International Application claims priority of U.S. Provisional Application No. 60/126,539, filed March 26, 1999 and U.S. Non-
5 Provisional Application No. 09/286,033, filed April 5, 1999. This invention was made, in part, with U.S. government support under Grant Contract Nos. HL-59157 and HL-24136 awarded by the National Institutes of Health. The United States government may have certain rights in this invention. Throughout this application various patents
10 and other publications are referenced. The disclosures of each and all of these patents and other publications in their entireties are hereby incorporated by reference into this application.

Field of the Invention

15

The invention generally relates to angiogenic factors and more particularly to the angiopoietin family of growth factors and to methods of decreasing or inhibiting vascular permeability and/or plasma leakage.

20

Background of the Invention

Plasma leakage, a key component of inflammation, occurs in a distinct subset of the microvessels. In particular, in most organs plasma
25 leakage occurs specifically in the venules. Unlike arterioles and capillaries, venules become leaky in response to numerous inflammatory mediators including histamine, bradykinin, and serotonin.

One characteristic of inflammation is the plasma leakage that results from intercellular gaps that form in the endothelium of venules. Most experimental models of inflammation indicate that these intercellular gaps occur between the endothelial cells of postcapillary and collecting
5 venules (Baluk, P., et al., Am. J. Pathol. 1998 152:1463-76). It has been shown that certain lectins may be used to reveal features of focal sites of plasma leakage, endothelial gaps, and finger-like processes at endothelial cell borders in inflamed venules (Thurston, G., et al., Am. J. Physiol, 1996, 271: H2547-62). In particular, plant lectins have been
10 used to visualize morphological changes at endothelial cell borders in inflamed venules of, for example, the rat trachea. Lectins, such as concanavalin A and ricin, that bind focally to inflamed venules reveal regions of the subendothelial vessel wall exposed by gaps that correspond to sites of plasma leakage (Thurston, G., et al., Am J
15 Physiol, 1996, 271: H2547-62).

The properties of the microvessels are dynamic. Chronic inflammatory diseases, for example, are associated with microvascular remodeling, including angiogenesis and microvessel enlargement. Microvessels can
20 also remodel by acquiring abnormal phenotypic properties. In a murine model of chronic airway inflammation, we found that airway capillaries acquire properties of venules, including widened vessel diameter, increased immunoreactivity for von Willebrand factor, and increased immunoreactivity for P-selectin. In addition, these
25 remodeled vessels leak in response to inflammatory mediators, whereas vessels in the same position in the airways of normal mice do not.

Certain substances have been shown to decrease or inhibit vascular permeability and/or plasma leakage. For example, mystixins are synthetic polypeptides that have been reported to inhibit plasma leakage without blocking endothelial gap formation (Baluk, P., et al., J. Pharmacol. Exp. Ther., 1998, 284: 693-9). Also, the beta 2-adrenergic receptor agonist formoterol reduces microvascular leakage by inhibiting endothelial gap formation (Baluk, P. and McDonald, D.M., Am. J. Physiol., 1994, 266:L461-8).

10 What factors determine whether a vessel will acquire phenotypic features of venules? One apparent clue came from studies of angiopoietin-1 (Ang1), a ligand for the endothelial cell-specific receptor TIE2. In mice that transgenically overexpress Ang1 in the skin under the keratin-14 promoter (K14-Ang1 mice), microvessels in the
15 position of capillaries have widened vessel diameter, immunoreactivity for P-selectin, and immunoreactivity for von Willebrand factor. Thus, these vessels have phenotypic features of venules.

Chronic inflammation is associated with blood vessel formation and
20 enlargement and changes in vessel phenotype. In mice with chronic airway inflammation, strain-dependent differences to the same stimulus have been shown to result in either blood vessel proliferation or enlargement, depending on the host response (Thurston, G., et al., Am. J. Pathol., 1998, 153: 1099-112). Analyses of mouse embryos deficient
25 in the TIE2 receptor illustrate its importance in angiogenesis, particularly for vascular network formation in endothelial cells. Sato, T.N., et al., Nature 376:70-74 (1995). In the mature vascular system,

the TIEs could function in endothelial cell survival, maintenance and response to pathogenic influences.

It has been suggested that the TIE receptors play roles in endothelial cell determination, proliferation, differentiation and cell migration and patterning into vascular elements. The predominant expression of the TIE receptors in vascular endothelia suggests that the TIEs play a role in the development and maintenance of the vascular system. The TIE receptors are also expressed in primitive hematopoietic stem cells, B cells and a subset of megakaryocytic cells, thus suggesting the role of ligands which bind these receptors in early hematopoiesis, in the differentiation and/or proliferation of B cells, and in the megakaryocytic differentiation pathway. Iwama, et al. Biochem. Biophys. Research Communications 195:301-309 (1993); Hashiyama, et al. Blood 87:93-101 (1996); Batard, et al. Blood 87:2212-2220 (1996).

An angiogenic factor, which was originally called TIE2 ligand-1 (TL1) but is also referred to as angiopoietin-1 (Ang1), has been identified that signals through the TIE2 receptor and is essential for normal vascular development in the mouse. By homology screening, an Ang1 relative has been identified and called TIE2 ligand-2 (TL2) or angiopoietin-2 (Ang-2). Ang-2 is a naturally occurring antagonist for Ang1 and the TIE2 receptor. For a description of the cloning and sequencing of TL1 (Ang1) and TL2 (Ang-2) as well as for methods of making and uses thereof, reference is hereby made to PCT International Publication No. WO 96/11269 published 18 April 1996 and PCT International Publication No. WO 96/31598 published 10 October 1996 both in the name of Regeneron Pharmaceuticals, Inc.; and S. Davis, et al.,

Cell 87: 1161-1169 (1996) each of which is hereby incorporated by reference. Ang1* is a mutant form of Ang1 that comprises the N-terminal domain of Ang-2 fused to the coiled-coil domain and the fibrinogen domain of Ang1 and that has a Cys to Ser mutation at amino acid 245 (See PCT International Publication No. WO 98/05779 published 12 February 1998 in the name of Regeneron Pharmaceuticals, Inc. which is hereby incorporated by reference). Ang1* has been shown to be a potent agonist for the TIE2 receptor.

10 Including the above-described angiopoietins, Applicants have identified a family of several related angiogenic factors. These have been designated TIE2 ligand-1 (TL1) also referred to as angiopoietin-1 (Ang1); TIE2 ligand-2 (TL2) or angiopoietin-2 (Ang-2); Tie ligand-3 (TL3) or angiopoietin-3 (Ang3) and Tie ligand-4 (TL4) or angiopoietin-4 (Ang4). For descriptions of the structural and functional properties of these four related factors, reference is hereby made to the following publications, each of which is hereby incorporated by reference: U.S. Patent No. 5,643,755, issued 7/1/97 to Davis, et al.; U.S. Patent No. 5,521,073, issued 5/28/96 to Davis, et al.; U.S. Patent No. 5,650,490, issued 7/22/97 to Davis, et al.; U.S. Patent No. 5,879,672, issued March 9, 1999; U.S. Patent No. 5,814,464, issued September 29, 1998; U.S. Patent No. 5,851,797, issued December 22, 1998; PCT International Application No. PCT/US95/12935, filed Oct. 6, 1995, published on April 18, 1996, with Publication No. WO 96/11269; and PCT International Application No. PCT/US96/04806, filed April 5, 1996, published on October 10, 1996, with Publication No. WO96/31598, both PCT applications in the name of Regeneron Pharmaceuticals, Inc.

The angiopoietins can be structurally divided into three domains: an N-terminal region lacking homology to any known structures; an alpha-helical-rich coil-coil segment similar to motifs found in many proteins that seem to promote multimerization; and a "fibrinogen-like domain" thus dubbed because it is distantly related by homology to a domain first found in fibrinogen but now noted to be in many other proteins (Davis, S. et al., (1996) Cell 87: 1161-1169). The fibrinogen-like domain represents the most conserved region of the angiopoietins, and recent studies indicate that it comprises the receptor-binding portion of the angiopoietins. In addition, all the information that determines whether an angiopoietin is an agonist or an antagonist of the TIE receptors appears to reside within the fibrinogen-like domain. For example, when chimeric molecules are made in which the fibrinogen-like domains of Ang1 and Ang-2 are swapped, agonistic or antagonistic abilities track with the fibrinogen-like domains. The N-terminal and coil-coil regions appear to serve mainly to multimerize the fibrinogen-like domains, which apparently must be clustered to be active. In fact, the N-terminal and coil-coil regions can be substituted with alternative motifs that allow clustering or multimerization. Thus, the activities of Ang1 and Ang-2 can be precisely mimicked by surrogates in which the fibrinogen-like domains (FD) of these factors are fused to the constant region of an antibody, resulting in FD-Fc fusions, which can then be clustered using secondary antibodies directed against the Fc. For a general description of the production and use of FD-Fc fusions, see International Publication Number WO 97/48804 published 24 December 1997. Using these techniques, one of skill in the art would be able to similarly make FD-Fc fusions using the fibrinogen-like domain of any angiopoietin family member. One practical advantage of

such surrogates is that native angiopoietins can be difficult to produce recombinantly, while the surrogates can be more easily produced.

As described in C. Suri, et al., Cell, 1996, 87: 1171-1180, the absence
5 of Ang1 causes severe vascular abnormalities in the developing mouse
embryo. Ang1 and Ang-2 have been described as naturally occurring
positive (agonist) and negative (antagonist) regulators of angiogenesis.
Positive or negative regulation of TIE2 is likely to result in different
outcomes depending on the combination of simultaneously acting
10 angiogenic signals.

The angiopoietins and members of the vascular endothelial growth
factor (VEGF) family are the only growth factors thought to be largely
specific for vascular endothelial cells. Targeted gene inactivation
15 studies in mice have shown that VEGF is necessary for the early stages
of vascular development and that Ang1 is required for later stages of
vascular remodeling. It has been reported that transgenic
overexpression of Ang1 in the skin of mice produces larger, more
numerous, and more highly branched vessels, however the
20 characteristics of the resultant vessels are largely unknown (Suri, C.,
et al., Science, 1998, 282:468-471). The present invention is the result
of Applicants' efforts to examine these vessels in greater detail by,
among other studies, assaying for vessel permeability/plasma leakage.

25 Summary of the Invention

The present invention provides for a method of decreasing or inhibiting
vascular permeability and/or plasma leakage in a mammal comprising

administering to the mammal a TIE2 receptor activator (agonist). By way of example, but not by way of limitation, the vascular permeability/plasma leakage may be produced by an inflammatory agent or by injury. In one embodiment of the invention, the mammal is a human. By vascular permeability, what is meant is any process that leads to leakage or extravasation of plasma including, but not limited to, increased endothelial permeability. See for example, McDonald, D.M., et al., *Microcirculation* 6: 7-22 (1999); Feng, D., et al., *Microcirculation* 6: 23-44 (1999); and Michel, C.C., and Neal, C.R., *Microcirculation* 6: 45-54 (1999), each of which is incorporated by reference herein in its entirety.

The invention further provides for a method wherein the TIE2 receptor activator is Ang1, or a polypeptide comprising a fragment or derivative thereof capable of activating the TIE2 receptor.

The invention also provides for a method wherein the TIE2 receptor activator is an activating antibody, or a polypeptide comprising a fragment or derivative thereof capable of activating the TIE2 receptor.

The invention further provides for a method wherein the TIE2 receptor activator is a small molecule, or a fragment or derivative thereof capable of activating the TIE2 receptor.

The invention also provides for a method of decreasing or inhibiting vascular permeability and/or plasma leakage in a mammal comprising administering to the mammal an Ang-2 inactivator such as an anti-Ang-2 neutralizing antibody. In one embodiment of this invention, the

mammal is a human. The invention also provides for a method wherein the anti-Ang-2 neutralizing antibody is a monoclonal antibody, including a wholly human monoclonal antibody. The invention further provides for a method wherein the anti-Ang-2 neutralizing antibody is a polyclonal antiserum. Alternatively the Ang-2 inactivator could be a small molecule.

The invention also provides for a composition comprising an Ang-2 inactivator in a vehicle, as well as a method of regulating angiogenesis in a patient comprising administering to the patient an effective amount of the composition. In one embodiment, the Ang-2 inactivator is a small molecule. The invention also provides for a composition comprising an anti-Ang-2 neutralizing antibody in a vehicle, as well as a method of regulating angiogenesis in a patient comprising administering to the patient an effective amount of the composition.

Brief Description of the Figures

FIGURE 1 - Ricin Stain Showing Vessels in Ang1 Over-expressing Mice are resistant to Mustard Oil Induced Vascular Leakage - The microvasculature of mice over-expressing Ang1 in the skin (K14-Ang1 - bottom panel) and wild-type control FVB/N mice (control - top panel) was stained by perfusion of lectin (ricin) after treatment with the inflammatory agent mustard oil, then examined in whole mounts of the ear skin.

FIGURE 2 - Leakage From Ear Vessels of Wild-Type and K14-Ang1 Mice - Mustard oil (5%) was applied to surface of ear skin, control ear

received no treatment. Serotonin (10 µg/ml) was injected dermally, control ear received injection of vehicle. Leakage was measured at 30 min after stimulus, n= 4 to 6 ears per group, values are mean ± SE.

*Significantly different from corresponding wild-type value, $P < 0.05$,

5 Bonferroni/Dunn test.

FIGURE 3 - Amount of extravasation of Evans blue in ears of wild-type, K14-Ang1, K14-VEGF, and K14-Ang1/VEGF mice 30 minutes after topical application of mustard oil. Asterisk marks significantly
10 greater leakage in untreated ears of K14-VEGF mice than in untreated ears of wild-type, K14-Ang1, or K14-Ang1/VEGF mice. Cross marks significantly greater leakage in treated ears of wild-type mice than in treated ears of K14-Ang1 or K14-Ang1/VEGF mice. # marks
15 significantly greater leakage in treated ears of K14-VEGF mice than in treated ears of K14-Ang1 or K14-Ang1/VEGF mice.

FIGURE 4 - Amount of extravasation of Evans blue in ears of wild-type mice injected intravenously with adenovirus expressing Ang1* or adenovirus expressing green fluorescent protein six days earlier, and
20 then treated for 30 minutes by topical application of mustard oil.

FIGURE 5 - Quantitation of tissue levels of Evans blue dye in ears from mice pre-treated systemically with either Ad-Ang1 or Ad-GFP, and 5-7 days later challenged with intradermal injections of VEGF protein or
25 saline (sterile solution of 0.9% NaCl) control; mustard oil-treated ears serve as an additional control. Also shown is blockage of the VEGF effect using co-injection of a specific VEGF antagonist, i.e. a 10-fold molar excess of a soluble VEGFR1-Fc fusion protein.

*, Significantly greater leakage than in control and Ad-Ang1 treated mice.

Detailed Description of the Invention

5

As described in greater detail below, Applicants have invented a method of decreasing or inhibiting vascular permeability and/or plasma leakage in a mammal comprising administering to the mammal a TIE2 receptor activator. By way of example, but not by way of limitation,
10 the vascular permeability/plasma leakage may be produced by an inflammatory agent or by injury. In one embodiment of the invention, the mammal is a human.

The invention further provides for a method wherein the TIE2 receptor
15 activator is Ang1, or a polypeptide comprising a fragment or derivative thereof capable of activating the TIE2 receptor.

The invention also provides for a method wherein the TIE2 receptor
20 activator is an activating antibody, or a polypeptide comprising a fragment or derivative thereof capable of activating the TIE2 receptor.

The invention further provides for a method wherein the TIE2 receptor
activator is a small molecule, or a fragment or derivative thereof
capable of activating the TIE2 receptor.

25

The invention also provides for a method of decreasing or inhibiting vascular permeability and/or plasma leakage in a mammal comprising administering to the mammal an Ang-2 inactivator such as an anti-

Ang-2 neutralizing antibody. In one embodiment of this invention, the mammal is a human. The invention also provides for a method wherein the anti-Ang-2 neutralizing antibody is a monoclonal antibody, including a wholly human monoclonal antibody. The invention further
5 provides for a method wherein the anti-Ang-2 neutralizing antibody is a polyclonal antiserum. Alternatively the Ang-2 inactivator could be a small molecule.

The invention also provides for a composition comprising an Ang-2
10 inactivator in a vehicle, as well as a method of regulating angiogenesis in a patient comprising administering to the patient an effective amount of the composition. In one embodiment, the Ang-2 inactivator is a small molecule. The invention also provides for a composition comprising an anti-Ang-2 neutralizing antibody in a vehicle, as well as
15 a method of regulating angiogenesis in a patient comprising administering to the patient an effective amount of the composition.

By way of example, but not limitation, the method of the invention may be useful in treating clinical conditions that are characterized by
20 plasma leakage/vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such as psoriasis or arthritis, including rheumatoid arthritis; asthma; generalized edema associated with burns; ascites and pleural effusion associated with tumors, inflammation or
25 trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age-related macular degeneration and diabetic retinopathy.

The compositions of the invention may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, 5 intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

For preparation of monoclonal antibodies, any technique which provides 10 for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique 15 to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

The monoclonal antibodies may be human monoclonal antibodies or 20 chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:7308-7312; Kozbor et al., 1983, *Immunology Today* 4:72-79; Olsson et al., 1982, *Meth. Enzymol.* 92:3-16). Chimeric antibody 25 molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, *Proc. Natl. Acad. Sci. U.S.A.* 81:6851, Takeda et al., 1985, *Nature* 314:452). Wholly human monoclonal antibodies are also provided for and can be made as

described in, for example, US Patent No. 5,939,598, issued August 17, 1999 and assigned to Abgenix, Inc.

Various procedures known in the art may be used for the production of polyclonal antibodies. For the production of Ang-2 inactivating antibodies, various host animals, including but not limited to rabbits, mice and rats can be immunized by injection with Ang-2, or a fragment or derivative thereof. For the production of TIE2 activating antibodies, various host animals, including but not limited to rabbits, mice and rats can be immunized by injection with TIE2 receptor extracellular domain, or a fragment or derivative thereof. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, polypeptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

A molecular clone of an antibody to a selected epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments which

contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by
5 reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high
10 performance liquid chromatography), or a combination thereof.

In alternative embodiments of the invention, a single chain Fv made be engineered. A single chain Fv (scFv) is a truncated Fab having only the V region of a heavy chain linked by a stretch of synthetic peptide to a V
15 region of a light chain. See, for example, US Patent Nos. 5,565,332; 5,733,743; 5,837,242; 5,858,657; and 5,871,907 assigned to Cambridge Antibody Technology Limited incorporated by reference herein.

The following examples are offered by way of illustration and not by
20 way of limitation.

Examples

Example 1:

25

It was previously shown that transgenic overexpression of Ang1 in the skin of mice (K14-Ang1) as described by Suri, C., et al., 1998, Science 282: 468-471, hereby incorporated by reference in its entirety,

produces larger, more numerous, and more highly branched vessels. The microvasculature of mice over-expressing Ang1 in the skin (K14-Ang1) and wild-type control FVB/N mice was then stained by perfusion of lectin, as generally described in Thurston, G. et al., 1998, Am. J. Pathol. 153(4):1099-112, hereby incorporated by reference in its entirety, then examined in whole mounts of the ear skin as shown in Figure 1.

Plasma leakage from ear vessels was measured in some mice after topical application of the inflammatory agents mustard oil or serotonin (See Inoue, H, et al., 1997, Eur. J. Pharmacol. 333:231-40) with the tracer Evans blue (See Baluk, P., et al., 1997, Am. J. Physiol. 272:L155-170). Plasma leakage was measured using the tracer dye Evans blue. Evans blue (EM Sciences, Cherry Hill, NJ) 30 mg/kg in a volume of 100 μ l was injected into one femoral vein of anesthetized mice, and 1 min later mustard oil or serotonin was administered to one ear. Mustard oil (Sigma, St. Louis, MO) was diluted to 5% in mineral oil and applied with a Q-tip to the dorsal and ventral surfaces of the skin of one ear, and nothing was done to the other ear (baseline control). Serotonin (Sigma) was made up at 0.22 mg/ml in sterile saline plus 0.005 N acetic acid (vehicle). Approximately 10 μ l was injected intradermally into the dorsal ear skin, and a similar volume of vehicle was injected into the other ear (control). Thirty minutes after the stimulus, the vasculature was perfusion-fixed (1% paraformaldehyde in 50 mM citrate buffer, pH 3.5) for 2 min. Ears were removed, blotted dry, and weighed. Evans blue was extracted from the ears with formamide and measured with a spectrophotometer at 610 nm.

We found vessels in the position of capillaries in the skin of K14-Ang1 mice were abnormally large and had phenotypic properties of venules, including strong immunoreactivity for P-selectin and von Willebrand factor. By comparison, in wild type mice the immunoreactivity of these proteins was largely restricted to venules. The microvasculature of ear skin was not leaky in either group of mice under baseline conditions (6.7 ± 1.7 ng of Evans blue per mg wet tissue weight in K14-Ang1 mice compared to 6.4 ± 1.6 ng/mg in wild-type mice), but was significantly less leaky in K14-Ang1 mice compared to control after treatment with mustard oil (5.9 ± 1.8 ng/mg in K14-Ang1 vs 18.1 ± 3.9 ng/mg in wild-type mice) - See Figure 2, top panel. Vessels in K14-Ang1 mice were also resistant to leakage after treatment with serotonin - See Figure 2, bottom panel. Although overexpression of Ang1 induced some of the phenotypic features of venules, skin vessels in K14-Ang1 mice are more resistant to leakage induced by inflammatory stimuli.

In summary, we found that the inflammatory mediator mustard oil induced plasma leakage in the ear skin of wild-type mice. However, contrary to our expectations, we found that mustard oil did not induce significant leakage in K14-Ang1 transgenic mice (Figure 2).

Furthermore, we found that another inflammatory mediator, serotonin, also failed to induce significant leakage in K14-Ang1 mice (Figure 2).

Example 2:

25

In another set of experiments, we found that skin vessels in mice that overexpress VEGF have increased baseline leakage. The vessels in these mice showed further increases in leakage after stimulation with

mustard oil. However, unexpectedly, the high baseline leakage in K14-VEGF transgenic mice was reduced to normal values in double transgenic K14-VEGF/Ang1 mice (Figure 3). In addition, mustard oil did not induce significant leakage in double transgenic K14-VEGF/Ang1 mice (Figure 3). These experiments establish that transgenic overexpression of Ang1 results in vessels that are resistant to leakage induced by inflammatory mediators (mustard oil, serotonin) and by transgenic overexpression of VEGF.

10 To determine whether acute administration of Ang1 can also reduce inflammation-associated leakage, we used an adenovirus encoding for angiopoietin-1* (adeno-Ang1*) to produce high systemic levels in otherwise normal mice. Ang1* has been shown to be a potent agonist for the TIE2 receptor. Plasma leakage was tested in ear skin of mice given this virus by iv injection. Control mice received iv injection of adenovirus encoding for a non-active protein (green fluorescent protein - GFP). At 6 days after administration of adenovirus, the amount of plasma leakage induced by mustard oil was significantly less in mice given adeno-Ang1* than in those given adeno-GFP (Figure 4). This experiment establishes that administration of Ang1 to wild-type mice can result in vessels that are resistant to inflammation-induced leak.

Example 3:

25 As stated *supra*, transgenic overexpression of VEGF led to the formation of abnormally leaky vessels, but the leakiness could be normalized by simultaneous transgenic overexpression of Ang1. To determine whether pre-administration of Ad-Ang1 could block leak

induced by VEGF in the adult setting, we injected VEGF protein intradermally into the ears of mice that we had previously injected intravenously with Evans blue dye. Dye extravasation resulted, and this extravasation could be blocked by co-administration of a VEGF inhibitor (Fig. 5). Pre-administration of Ad-Ang1 but not Ad-GFP also blocked the leakage induced by VEGF (Fig. 5), demonstrating that short-term delivery of Ang1 could make adult vessels resistant to vascular leak induced by either an inflammatory agent or VEGF.

Evans Blue assays were performed as recently described (Thurston, G. *et al.*, *Science* **286**, 2511-2514 (1999); Suri, C. *et al.*, *Science* **282**, 468-471 (1998); Thurston, G., Baluk, P., Hirata, A. & McDonald, D.M., *Am J Physiol* **271**, H2547-2562 (1996)). In addition, the modified Miles assay in adult mice was performed by injecting Evans blue, followed one minute later by an intradermal injection of VEGF (400 nanograms in 8 microliters) into the dorsal aspect of the middle region of the ear; ears were harvested thirty minutes after injection and processed as in other Evans blue assays. To test the effects of Ang1 in the modified Miles assays and controls depicted in Fig. 5, the mice were injected with 109 PFU of Ad-Ang1 or Ad-GFP 1 to 5 days prior to the assay.

The above studies demonstrate that acute administration of Ang1 protects the adult vasculature against leak induced by a variety of challenges, as was the case with Ang1 transgenically over-expressed during vessel development.

It has long been appreciated that VEGF can rapidly increase vascular permeability in the adult, and that VEGF-induced plasma leakage may

contribute to a variety of disease processes. Our data demonstrate, for the first time, that this potentially adverse effect of VEGF can be blocked by acute administration of Ang1.

- 5 The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within
- 10 the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal a TIE2 receptor activator.
2. The method of claim 1, wherein the mammal is a human.
3. The method of claim 1, wherein the TIE2 receptor activator is Ang1, or a polypeptide comprising a fragment or derivative thereof capable of activating the TIE2 receptor.
4. The method of claim 1, wherein the TIE2 receptor activator is an activating antibody, or a polypeptide comprising a fragment or derivative thereof capable of activating the TIE2 receptor.
5. The method of claim 1, wherein the TIE2 receptor activator is a small molecule, or a fragment or derivative thereof capable of activating the TIE2 receptor.
6. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal an anti-Ang-2 neutralizing antibody.
7. The method of claim 6, wherein the mammal is a human.
8. The method of claim 6, wherein the anti-Ang-2 neutralizing antibody is a monoclonal antibody.

9. The method of claim 8 wherein the monoclonal antibody is a wholly human monoclonal antibody.
10. The method of claim 6, wherein the anti-Ang-2-neutralizing antibody is a polyclonal antiserum.

1/5

Figure 1

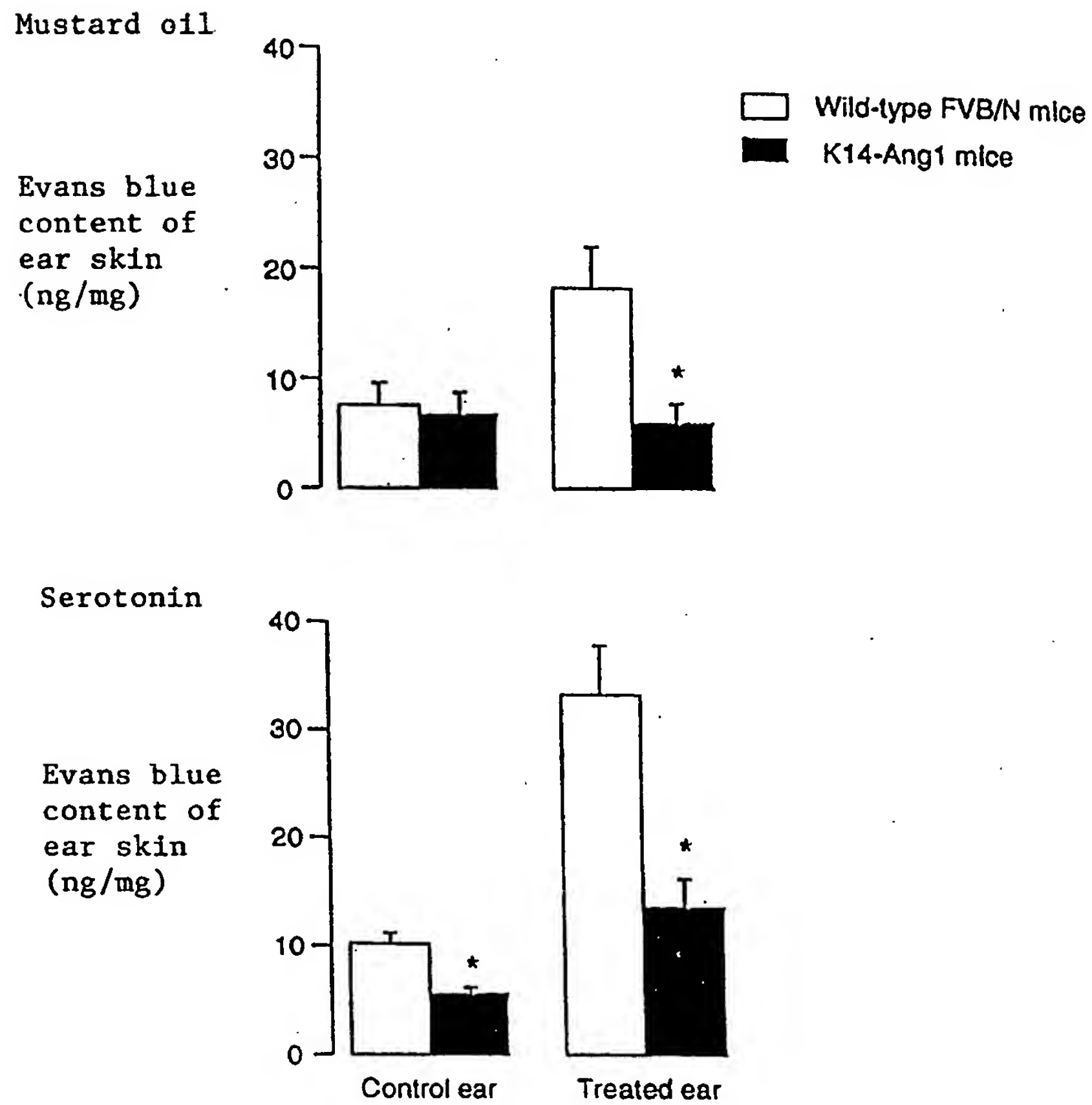
Control



K14-Ang1

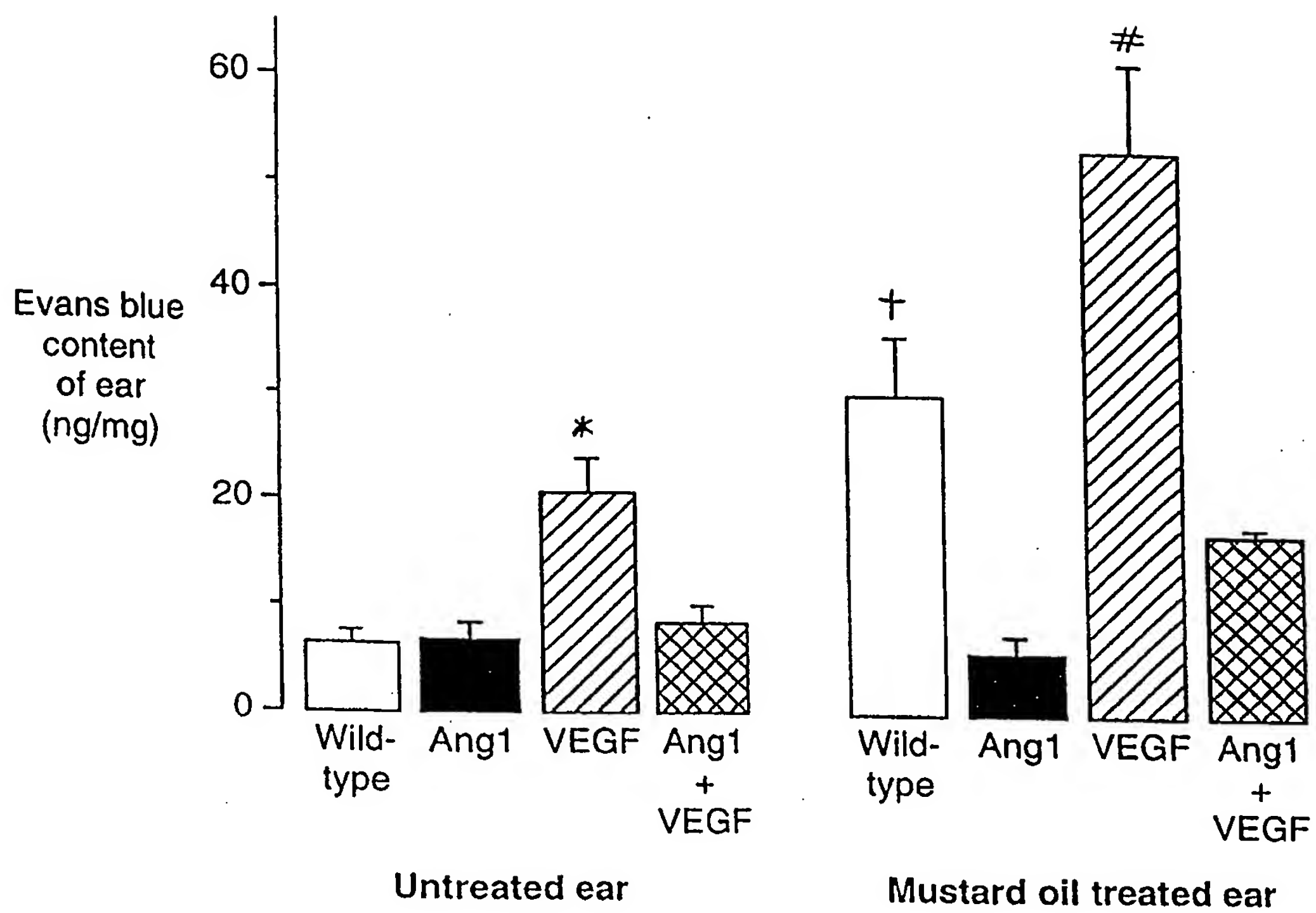


2/5
Figure 2.



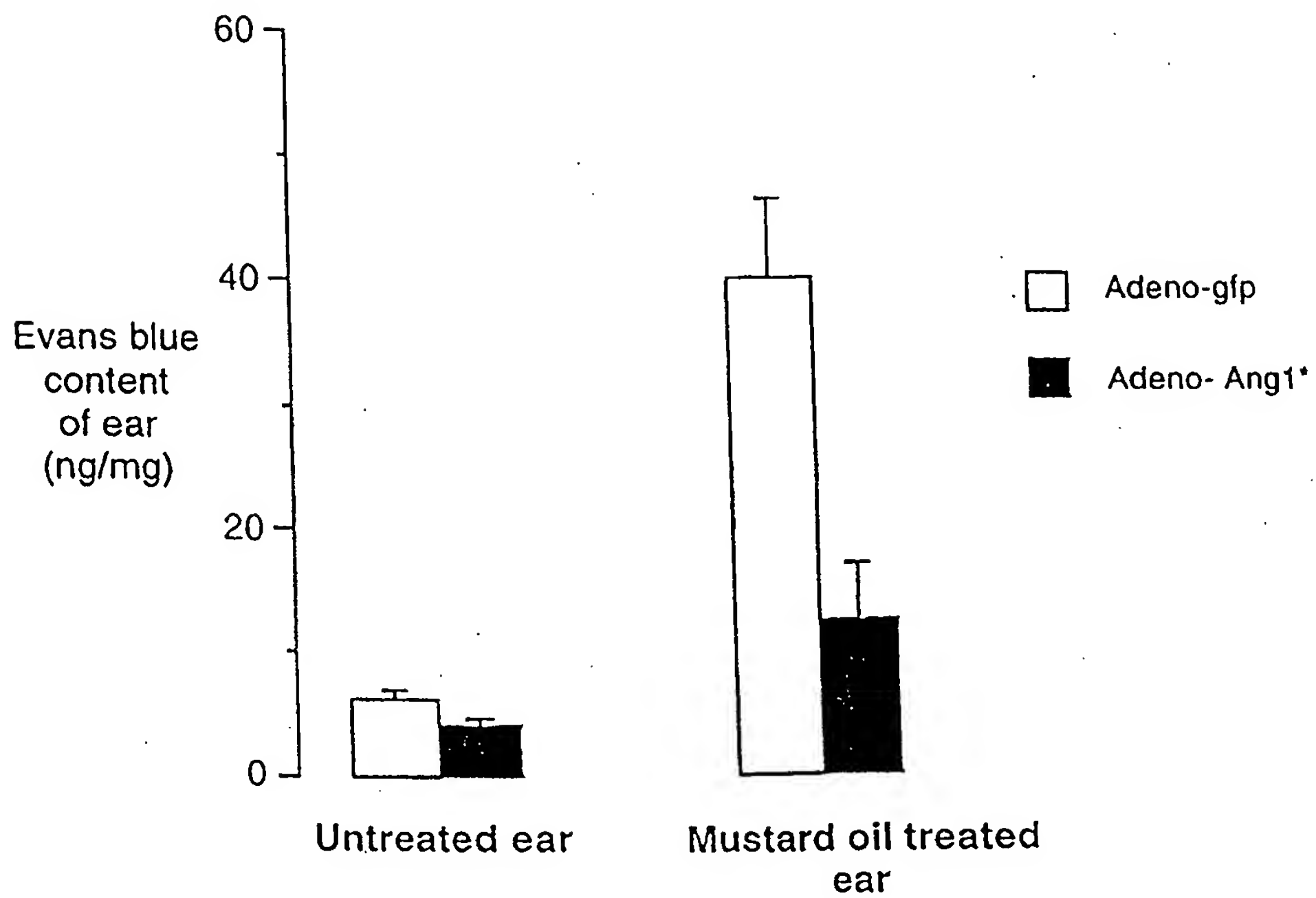
3/5

Figure 3



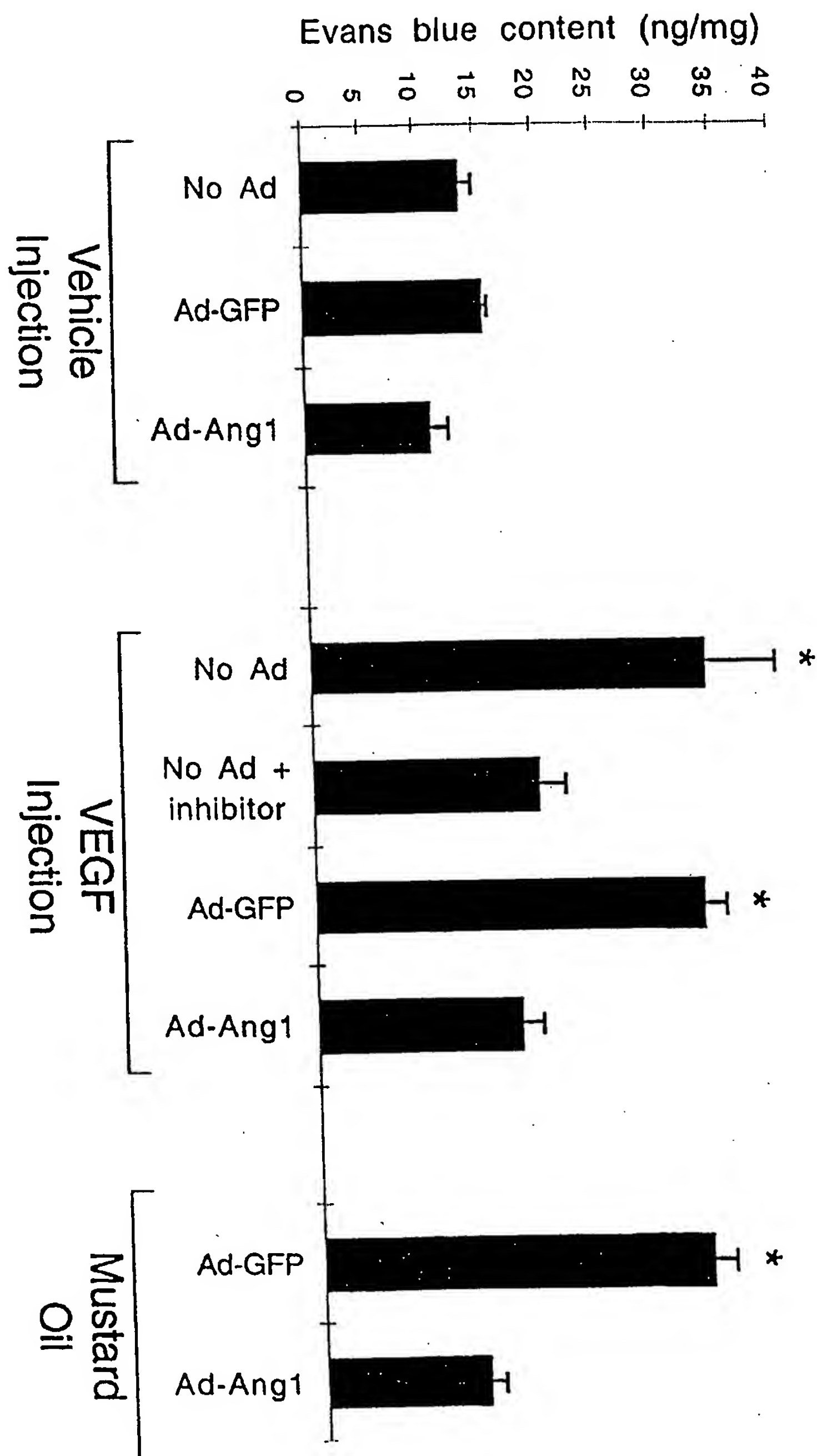
4/5

Figure 4



5/5

Figure 5



INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 00/07336

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/17 A61K38/18 A61K39/395 A61P9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, CANCERLIT, AIDSLINE, LIFESCIENCES, EMBASE, CHEM ABS Data, SCISEARCH, BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 482 930 A (WEI EDWARD T ET AL) 9 January 1996 (1996-01-09) column 1, line 19-34 column 3, line 41-54 claims	1
X	PATENT ABSTRACTS OF JAPAN vol. 011, no. 273 (C-445), 4 September 1987 (1987-09-04) -& JP 62 077318 A (EISAI CO LTD), 9 April 1987 (1987-04-09) abstract	1



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

31 July 2000

Date of mailing of the international search report

18/08/2000

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/07336

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; DVORAK H F ET AL: "Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis." retrieved from STN Database accession no. 1999109226 XP002143874 abstract & CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, (1999) 237 97-132. REF: 171 ,</p>	1-10
A	<p>MAISONPIERRE PETER C ET AL: "Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis." SCIENCE (WASHINGTON D C), vol. 277, no. 5322, 1997, pages 55-60, XP002143872 ISSN: 0036-8075 the whole document</p>	1-10
A	<p>WO 98 07832 A (LUDWIG INSTITUTE FOR CANCER RESEARCH, USA;HELSINKI UNIVERSITY LICENSIN) 26 February 1998 (1998-02-26) cited in the application page 6, line 28 -page 10, line 18 example 3</p>	1-10
P,A	<p>WO 00 18804 A (SMITHKLINE BEECHAM CORPORATION, USA;SMITHKLINE BEECHAM PLC) 6 April 2000 (2000-04-06) page 3, line 22-26 page 19, line 29 -page 20, line 6</p>	1-10
T	<p>THURSTON GAVIN ET AL: "Angiopoietin-1 protects the adult vasculature against plasma leakage." NATURE MEDICINE, vol. 6, no. 4, April 2000 (2000-04), pages 460-463, XP002143873 ISSN: 1078-8956 the whole document</p>	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/07336

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WO 9807832 A	26-02-1998	AU 4079697 A EP 0956339 A	06-03-1998 17-11-1999
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